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A conserved domain of herpes simplex virus ICP34.5 regulates protein phosphatase complex in mammalian cells

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Abstract ICP34.5, encoded by herpes simplex virus 1, is a protein phosphatase 1 (PP1) regulatory subunit that mediates dephosphorylation of the α subunit of translation initiation factor 2 (eIF2α). However, the mechanism of its action remains poorly understood. Here, we show that amino acid substitutions in the arginine-rich motif have differential effects on ICP34.5 activity. The phenotypes parallel with viral protein synthesis and cytopathic effects in virus infected cells. Besides the consensus PP1 binding motif, the Arg-motif appears to enhance the interaction between ICP34.5 and PP1. These results suggest that concerted action between the PP1 binding domain and the effector domain of ICP34.5 is crucial for eIF2α dephosphorylation and viral protein synthesis.

Keywords: ICP34.5; PP1; eIF2α; Arginine rich motif

1. Introduction

ICP34.5, a protein encoded by $\gamma_1$34.5 gene of herpes simplex viruses (HSV), is essential for replication in vivo [1–3]. ICP34.5 antagonizes the host response mediated by the double-stranded RNA dependent protein kinase (PKR) in the early stage of in infection and facilitates viral egress in the late stage of infection [8]. In infected cells, HSV DNA replication activates PKR, which phosphorylates the α subunit of translation initiation factor 2 (eIF2α) and shuts off viral protein synthesis [4]. As a countermeasure, ICP34.5 is expressed to recruit cellular protein phosphatase 1 (PP1) that dephosphorylates eIF2α [5,6]. As a result, the efficient viral replication and productive viral infection proceed.

ICP34.5 consists of an amino-terminal domain, a linker region of triplet repeats (Ala-Thr-Pro), and a carboxyl-terminal domain [6,7]. The amino terminal domain is implicated in virus maturation, by a yet unclear mechanism [8]. The linker region, with a varying number of the triplet repeats, modulates neuroinvasion [9]. The carboxyl terminal domain functions to counteract the PKR response by host cells [6,10]. This portion of the viral protein is homologous to the corresponding domain of the cellular protein GADD34 expressed under stress conditions, such as DNA damage, apoptosis and ER stress, and involved in the negative regulation of a stress-inducible gene CHOP [11–14]. The carboxyl terminus of ICP34.5, functionally interchangeable with that of GADD34, binds and redirects PP1 to eIF2α [6,15]. Site-specific mutations in the PP1 binding domain, with a signature motif present in other PP1 interacting proteins, abolish eIF2α dephosphorylation and impairs viral replication [5,16,17]. In addition, mutations in the putative effector domain disrupt the activity of ICP34.5 [16,18,19]. However, the role of the effector domain in this process remains unknown.

In this work, we have characterized an Arg rich region with a stretch of conserved amino acids distal to the PP1 binding domain. We report that Arg residues in this region have differential effects on eIF2α dephosphorylation by PP1 both in vitro and in vivo. We also demonstrate that viral protein synthesis and cytopathic effects parallel with the extent of eIF2α dephosphorylation in virus infected cells. Our data leads to the point that the cooperation of PP1 binding domain and effector domain is of critical importance to the biochemical and biological functions of ICP34.5 to promote viral protein synthesis and effective infection.

2. Materials and methods

2.1. Cells and virus

Vero, Hela, HEK293T and NIH3T3 cell lines were obtained from the American Type Culture Collection and propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hela and HEK293T) or 10% new born calf serum (Vero and NIH3T3). Recombinant virus R3616 lacks a 1 kb fragment encompassing the $\gamma_1$34.5 gene [1].

2.2. Plasmids

Plasmid encoding human PP1c, pCMVneoPP1CS1α, was a gift from Dr. Anna DePaoli-Roach (Indiana University School of Medicine, IN). The ICP34.5 mutants were derived from pRB143 which contains the BamHI S fragment of HSV-1(F) DNA. For bimolecular fluorescence

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complementation (BiFC) assay [20]. DNA fragments encoding ICP34.5 WT, 3A, R255D, EDD, ED, and $V^{193}E$, $F^{195}L$ were fused to the nucleotide encoding the carboxyl-terminal fragment containing residues 155–238 (YC) of YFP, respectively. The PP1 coding region was fused to sequences encoding residues 1–154 (YN) of YFP. The fused regions were connected by linker sequences as recommended [20].

2.3. Immunoblot analysis

Cells were harvested and lysed in buffer containing 50 mM Tris–HCl (pH 8.0),150 mM NaCl, 0.5% deoxycholic acid, 0.1% SDS, 1% NP 40%, and protease inhibitor cocktail at 4 °C. After centrifugation, supernatants were subjected to SDS–PAGE and Western blot with antibodies against ICP34.5, HSV antigens (Dako Corporation), phosphorylated eIF2α-Ser-51 (Biosource Inc.), eIF2α (Cell Signaling Technology Inc.), or α-tubulin antibody (Sigma), as indicated respectively.

2.4. In vitro eIF2α dephosphorylation assay

GST-PKR fusion protein was expressed and purified from *Escherichia coli* BL21(DE3) strain as described previously [18]. Cytoplasmic S10 fractions were prepared from lysates of Hela cells transfected by the plasmids encoding wild-type ICP34.5 and the mutants as described previously [6]. To prepare phosphorylated eIF2α as substrate, eIF2 purified from rabbit reticulocytes was incubated with GST-PKR and 0.1 mM ATP in the reaction buffer containing 20 mM Tris–HCl (pH 7.5), 40 mM KCl, and 2 mM $MgCl_2$ for 30 min at 34 °C. Aliquots of the phosphorylated eIF2α were then reacted with S10 fractions in buffer containing 20 mM Tris–HCl (pH 7.5), 40 mM KCl, 2 mM $MgCl_2$, and 0.1 mM EDTA at 34 °C for 10 min. The reaction was stopped and samples were subjected to Western blot analysis with antibodies against eIF2α and eIF2α Ser-(P)-51, respectively.

2.5. Bimolecular fluorescence complementation (BiFC) assays

The BiFC method was adopted to investigate protein–protein binding in HeLa cells [20]. Briefly, constructs encoding fusion proteins, YC-34.5 variants and YN-PP1, were co-transfected into Hela cells grown on glass cover slips using Lipofectamine2000 (Invitrogen). At 48 h post-transfection, cell culture dishes were placed at room temperature for 1–3 h and stained with DAPI prior to microscopic analysis. The fluorescence emission was examined in living cells by confocal microscopy and images were processed using Olympus Fluoview FV1000. YFP fluorescence of living cells was also subjected to FACS analysis with excitation wavelength at 500 nm and emission wavelength at 535 nm.

2.6. Virus infection assays

Recombinant retroviruses were generated as described previously [21]. Briefly HEK293T cells were transfected with plasmid pHIV-34.5WT, pHIV-3A, pHIV-R255D, pHIV-EDD, pHIV-$V^{193}E$, $F^{195}L$, or HIV-GFP, along with pHIV-*trans* and pVSVG using calcium phosphate precipitation method. At 48-hour post-transfection, culture media containing the recombinant viral particles were collected to transduce NIH3T3 cells. Monolayers of transduced cells were either mock-infected or infected with γ_1*34.5 null* virus R3616. At an indicated time point after infection, cells were examined with a microscope or harvested to determine viral protein production by Western blot analysis [16].

3. Results

3.1. Effects of amino acid substitutions in ICP34.5 on eIF2α dephosphorylation

Sequence alignment was carried out with the carboxyl terminal domains derived from ICP34.5 of HSV type I and type II, and GADD34 of human, mouse, and hamster species

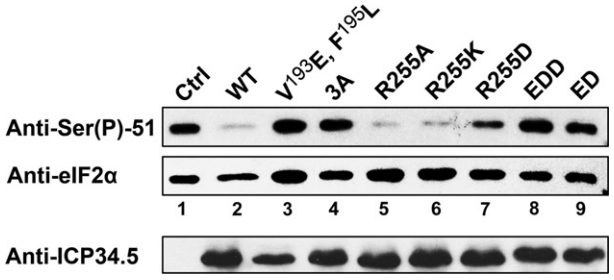

Fig. 2. Dephosphorylation of eIF2α in vitro. Purified eIF2α was phosphorylated at 34 °C by PKR for 30 min. An aliquot was added to reaction solutions containing the lysates of cells transfected plasmids encoding none (Ctrl), ICP34.5 (WT), or ICP34.5 mutants, respectively. Samples were incubated for 10 min at 34 °C, then subjected to SDS–PAGE, and Western blot analysis with antibodies against the phosphorylated eIF2α at Ser-51 (the top panel), eIF2α (the middle panel), and ICP34.5 (the bottom panel), respectively.

A

HSV-1	PATPA	RVRF	SPHVRVRHLVV	WASAARLARRGSWARERADR	ARFRRRVAEAEAVIGPCLGP	246
HSV-2	DAPRG	KVCF	SPRVQVRHLVA	WETAARLARRGSWARERADR	DRFRRRVAAAEAVIGPCLEP	229
Human	PLKAR	KVRF	SEKVTVHFLAV	WAGPAQAARQGPWEQLARDR	SRFARRITQAQEELSPCLTP	619
Mouse	PLKAR	KVHF	AEKVTVHFLAV	WAGPAQAARRGPWEQFARDR	SRFARRIAQAEEKLGPYLTP	612
Hamster	PLRAR	KVHF	SENVTVHFLAV	WAGPAQAARRGPWEQLARDR	SRFARRIAQAEEKLGPYLTP	558
Consensus	----------	KV--F	-S----V--V----L--V	WA---- A----ARRG--W---------- DR	--RF---RR -----AE---LGP--L--P	

HSV-1	EA	RARA LAR	GAGPANSV------	----------	----------	263
HSV-2	EA	RARA RAR	ARAHEDGGPAE	EEEAAAAARGSSAAAGPGRR	AV----------	261
Human	AA	RARAWAR	LRNPPLAP IPA	LTQTLPSSSVPSSPVQTTPL	SQAVATPSRSSAAAAAALDL	669
Mouse	DS	RARAWAR	LRNPSLPQSEP	RSSSEATPLTQDVTTPSPLP	SETPSPSLYLGGRRG----------	657
Hamster	AF	RARAWAR	LGNPSLPLALE	PICDHTFFPSQ----------	----------	590
Consensus	-----	RARA---AR	----------	----------	----------	

B

Mutation Names	Amino Acids
WT	. . . ^{192}RVRF . . . . . ^{248}ARARALARG . . . .
$V^{193}E$, $F^{195}L$	E L
3A	A A A
R225A	A
R255K	K
R255D	D
EDD	E D D
ED	E D

Fig. 1. (A) Amino acid sequence alignment of the carboxyl terminal domains of ICP34.5 from HSV-1 (AAA45790.1), HSV-2 (NP_044529), GADD34 proteins of Hamster (AAA36983), human (AAC25631.1), and mouse (NP_032680). The PP1 conserved sequences of binding and Arg rich motifs are underlined. (B) Site-specific mutants constructed in this work. Numbers denote amino acid positions.

(Fig. 1A). This revealed several highly conserved amino acids among these proteins. The region bearing the signature sequence (KVxF) represents the PP1 binding motif, and the region carboxyl terminal to this domain is tentatively defined as the effector domain. We focused on a conserved segment with a cluster of Arg and Ala, which spans amino acid 248–256. Site-specific mutations were designed based on size, polarity, and electric charge (Fig. 1B). Substitutions of amino acid were also introduced to the PP1 binding motif as a control. Cells were transiently transfected with plasmids expressing ICP34.5 variants and the lysates were processed for protein expression by Western blot analysis with anti-ICP34.5 antibody. Aliquots of lysate were subjected to in vitro dephosphorylation assay as described in Section 2. As expected, wild-type ICP34.5 (WT) mediated eIF2α dephosphorylation efficiently whereas the PP1 binding mutant ($V^{193}E$, $F^{195}L$) failed to do so (Fig. 2, lanes 2 and 3). As expected, the control lysate (Ctrl) did not facilitate eIF2α dephosphorylation (Fig. 2, lane 1). It appears that a basic residue or non-polar residue is tolerated at position 255, whereas, acidic residue substitution in Arg 255 by Asp marginally reduced eIF2α dephosphorylation (Fig. 2, lanes 5–7), suggesting that a negatively charged residue is only partially disruptive at this position. Substitutions in Arg-249 and Arg-251 with Glu and Asp, respectively (ED) completely impaired eIF2α dephosphorylation (Fig. 2, lane 9) to the same extent as an additional substitution of Arg-255

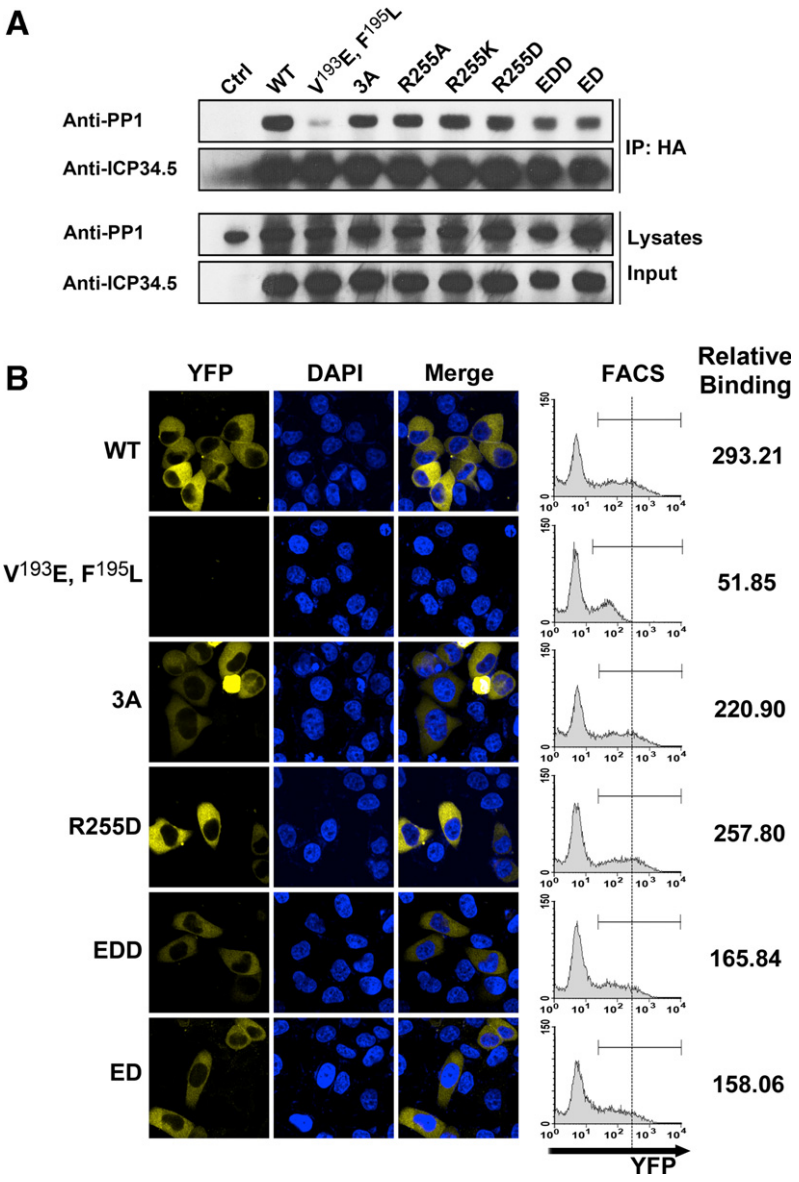

Fig. 3. Interaction between PP1 and ICP34.5 variants. (A) Co-immunoprecipitation of ICP34.5 mutants with PP1. HEK293T cells were transfected with plasmid encoding HA-tagged ICP34.5 variants or plasmid backbone as control(Ctrl), respectively. Cell lysates were incubated with anti-HA antibody and protein G-agarose. The co-precipitated proteins were resolved by Western blot analysis using antibodies against PP1 and ICP34.5 (upper two panels). Aliquots of lysates were also used to verify the expression of ICP34.5 variants and endogenous PP1 (lower two panels). (B) Visualization of ICP34.5 and PP1 interactions in live cells. Hela cells were co-transfected with plasmids encoding YFP fragments fused proteins, YN-PP1 and YC-ICP34.5 variants, respectively. At 48 h after transfection, cells were stained with DAPI and subjected to confocal fluorescence microscopy (left) and FACS analysis (middle). The mean values of YFP intensity (right) represent the relative binding efficiency of PP1-ICP34.5 variants.

with Asp (EDD) (Fig. 2, lane 8). Similarly, a triple substitution at Arg-249, Arg-251 and Arg-255 with Ala drastically reduced eIF2α dephosphorylation. These results suggest that Arg-249 and Arg-251 play indispensable role in ICP34.5 mediated PP1 activity towards eIF2α.

3.2. Interaction between protein phosphatase 1 and ICP34.5 variants

Since mutations in the Arg rich motif negatively affect eIF2α dephosphorylation, we examined whether this region contributes to PP1 binding. At first, we addressed this issue by co-immunoprecipitation. Specifically, cells were transfected with one of the plasmids encoding ICP34.5 variants. Lysates of cells were then analyzed for protein expression. As shown in Fig. 3A (upper panels), the co-immunoprecipitation experiment validated the binding of PP1 with ICP34.5 (WT), but not the PP1 binding mutant ($V^{193}E$, $F^{195}L$). It also revealed that, under this experimental condition, those mutations at Arg rich motif were capable of binding PP1 efficiently. The expression of ICP34.5 variants was verified to be comparable in Fig. 3A (lower panels).

We further investigated the interaction between PP1 and ICP34.5 in live cells by recently developed and widely accepted bimolecular fluorescence complementation (BiFC) assay. This method allows live detection of the biological interaction of two binding partners [20]. As described in Section 2, a pair of constructs encoding fusion proteins YN-PP1 and YC-34.5 or mutants was co-transfected into Hela cells and examined by confocal fluorescence microscopy. As shown in Fig. 3B (images), wild-type ICP34.5 (WT) associated with PP1 as illustrated by a strong fluorescent signal. In contrast, the PP1 binding mutant ($V^{193}E$, $F^{195}L$) was unable to interact with PP1 (Fig. 3B, the second row). Interestingly, mutants with amino acid substitutions in the Arg rich motif (3A, R255D, EDD, and ED) still associated with PP1 in the cytoplasm, suggesting that these mutants are capable of forming the ICP34.5-PP1 complexes. It is worth noting that despite the fact that ICP34.5 exists in both the nucleoli and cytoplasm [20], the interaction between PP1 and ICP34.5 was only seen in the cytoplasm.

To compare the binding effect of ICP34.5 variants with PP1, cells were subjected to flow cytometry analysis (Fig. 3B, histograms). The mean fluorescence intensity was measured (Fig. 3B, right column) as this parameter reflects the relative binding effect based on a long half-life of the associated fragments [20]. By comparison with the arbitrary baseline defined by the maximal fluorescence intensity of PP1 binding mutant ($V^{193}E$, $F^{195}L$) (Fig. 3B, the dotted vertical line across the histograms), it is evident that wild-type ICP34.5 efficiently formed a complex with PP1, with a score of 293.21. Likewise, ICP34.5 mutants with amino acid substitutions exhibited positive in complex formation. In this respect, R255D and 3A substitutions had modest effects, with binding scores of 220.9 and 257.8, respectively. In contrast, EDD and ED substitution resulted in a significant decrease, with binding scores of 165.8 and 158.06, respectively. Expression of each binding partners were examined to be equivalent (data not shown). Collectively, these results suggest that the Arg rich motif contributes to the efficiency of the ICP34.5-PP1 association.

3.3. The role of the Arg rich motif in viral protein synthesis

In infected cells, wild-type HSV, but not the *$\gamma_1$34.5 null* mutant R3616, prevents the shut-off of protein synthesis [4]. To further evaluate the effect of the Arg rich motif in a physiologically relevant system, we measured viral polypeptide synthesis compensated by the ICP34.5 variants. Specifically, NIH3T3 cells were transduced with recombinant retrovirus particles expressing ICP34.5 variants, and then challenged with R3616. The cell lysates were processed for Western blot analysis using antibodies specific to HSV proteins. As shown in Fig. 4A, viral protein synthesis in cells expressing wild-type ICP34.5 was of fourfold as that in cells expressing GFP or the PP1 binding mutant ($V^{193}E$, $F^{195}L$). Although R255D substitution had no effect, other mutations (3A, EDD, and ED) led to a 2–3-folds reduction in viral protein synthesis in comparison with wild-type ICP34.5 (Fig. 4A). In parallel, dephosphorylation of endogenous eIF2α correlated well with the viral protein synthesis profiles (Fig. 4B). Expression of ICP34.5 in retrovirus transduced cells was confirmed in Fig. 4C.

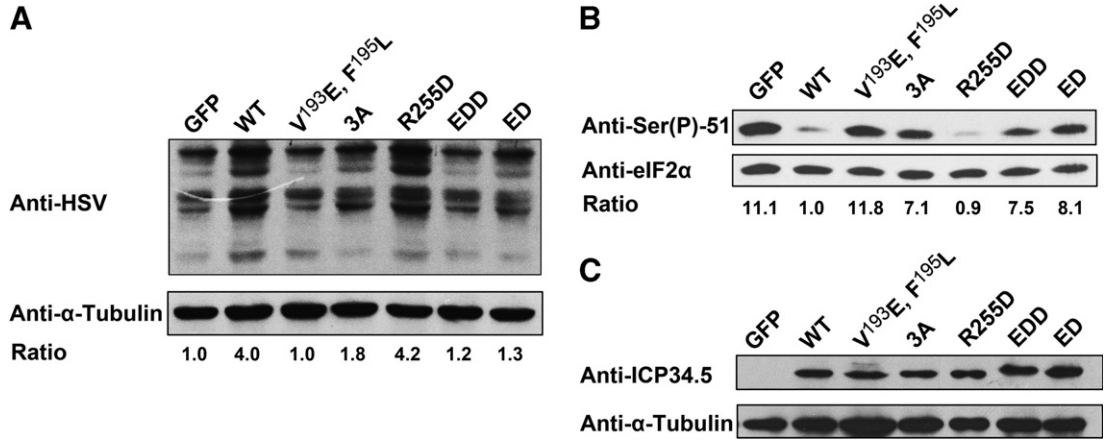

Fig. 4. Viral protein synthesis and endogenous eIF2α dephosphorylation. (A) NIH3T3 cells were transduced by retrovirus expressing GFP and indicated ICP34.5 variants, respectively. On day 5, cells were infected with the *$\gamma_1$34.5 null* virus R3616 at 10 pfu per cell for 12 h. Cells were harvested and lysates prepared for Western blot analysis with antibody against HSV-1 proteins and α-tubulin. Viral protein synthesis was quantitated by densitometry and presented as a ratio relative to α-tubulin. (B) Endogenous eIF2α and phosphorylated eIF2α were detected by Western blot with antibodies against eIF2α and phosphorylated eIF2α, respectively. (C) Expression of ICP34.5 variants in transduced cells was verified with anti-ICP34.5 antibody.

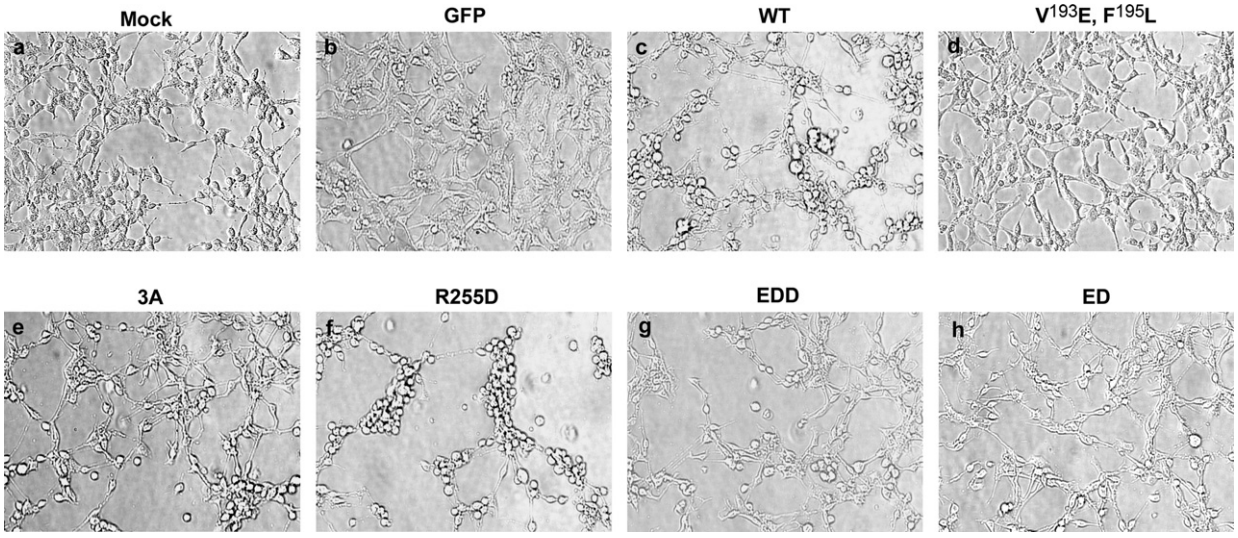

Fig. 5. Virus induced cytopathic effects. Monolayers of transduced NIH 3T3 cells were infected with the γ_1*34.5 null* mutant R3616 at 0.05 pfu per cell. At 48 h post-infection, cells were examined with a light microscope and images were captured.

3.4. Effects of ICP34.5 mutants on virus induced cytopathic effects

HSV infection typically induces morphological changes and consequently destroys infected cells due to active viral replication. To assess the biological significance of eIF2α dephosphorylation mediated by ICP34.5 variants, we also examined the cytopathic effects in virus-infected cells. As described above, NIH3T3 cells were transduced with various constructs and then mock infected or infected with the $\gamma_1$34.5 null virus R3616. At 48 h post-infection, the cells were examined by light microscopy. As illustrated in Fig. 5, mock-infected cells formed a monolayer, with most cells displaying spindle morphology (Fig. 5a). In cells expressing GFP or the PP1 binding mutant ($V^{193}E$, $F^{195}L$), the γ_1*34.5 null* virus did not cause any major changes as compared to mock-infected cells (Fig. 5, panels a, c and d). In contrast, cells expressing wild-type ICP34.5 or R255D exhibited dramatic changes as many cells became rounded up and formed clumps (Fig. 5, panels c and f). Notably, a large portion of cells were destroyed by virus infection where no intact cells were seen, indicating an efficient viral infection. In cells expressing 3A, EDD, or ED, the virus induced merely moderate cytopathic effects. Some cells exhibited cell rounding and clumping, indicative of a partial viral replication. Therefore, these cytopathic effects correlated well with the ability of ICP34.5 to mediate PP1 binding, eIF2α dephosphorylation, and viral protein synthesis.

4. Discussion

In mammalian cells, activated PKR induced by interferon phosphorylates eIF2α, leading to the shut-off of protein synthesis and thus the inhibition of viral replication. In this respect, many viruses, including herpes viruses, have evolved mechanisms to escape the antiviral response [22]. Previous studies demonstrate that evasion of the PKR response is essential for HSV infection, which involves the $\gamma_1$34.5 gene product [4,6,18]. This process requires the carboxyl terminus of the ICP34.5 protein that bears a PP1 binding motif and a putative effector domain [5,10,16].

Our analysis indicates that amino acid substitutions in the Arg rich motif of ICP34.5 affect eIF2α dephosphorylation. Furthermore, site-specific mutations in the Arg rich region of ICP34.5 appeared intact to PP1 binding in co-immunoprecipitation analysis, is consistent with that reported for GADD34 mutants [23]. However, the differential effect on PP1 binding was evident in live cells. These data raise the hypothetical model in which, while ICP34.5 determines the PP1 binding by the hydrophobic groove as that seen in other PP1 regulatory subunit, such as G_M [17], the Arg rich motif plays an accessory role in fine-tuning PP1 binding. Crystal structure of ICP34.5 is not yet available, but secondary structure prediction suggests that there are three α-helixes in the effector domain. Additional work is required to evaluate the impact of mutations on the structure of ICP34.5.

In HSV infected cells, ICP34.5 mediated eIF2α dephosphorylation renders viral polypeptide synthesis [6,16]. In this regard, the Arg rich motif is a functional element in the context of HSV infection. First, deficient protein synthesis in cells infected with γ_1*34.5-null* virus R3616 was rescued by expression of wild-type ICP34.5 or R255D, but not mutations in multiple Arg residues. Second, the above phenotypes correlate well with eIF2α dephosphorylation. Third, viral protein synthesis was mirrored by the extent of cytopathic effects seen in infected cells.

In summary, we demonstrated the correlation of biochemical and biological roles of the conserved Arg motif of ICP34.5 in HSV infection. We also addressed the differential effect of Arg mutants on PP1 binding, leading to the hypothesis that the cooperation of PP1 binding domain and effector domain is of critical importance to the function of ICP34.5. Further studies on three-dimensional structure would help to understand the mechanism.

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